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Chemistry and Function of Pectins

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A Critical Reexamination of Molecular Weight and Dimensions for Citrus Pectins

E-1000 and E-linear μ Bondagel[1] high performance size exclusion chromatography (HPSEC) columns were calibrated in root-mean square (RMS) radii of gyration (R_g) by using a combination of pullulan and dextran standards. By assuming that universal calibration applies, to pectins the number-average R_g in 0.05M and 0.1M NaCl for a series of pectins with varying degrees of esterification in the protonated and sodium forms were calculated from chromatograms. This procedure yields well-defined R_g averages that can be used readily to calculate molecular parameters for comparison with other methods. Such comparisons were not feasible when an earlier HPSEC method was used. Furthermore, by assuming rod-like structure for the pectins, number-average lengths ($\bar{l}_n$) and degree of polymerization ($\overline{DP}_n$) were calculated from $\bar{R}_{gn}$. Importantly, $\overline{DP}_n$ and $\bar{l}_n$ values from HPSEC fell between values from end group titration and membrane osmometry which is consistent with previous findings that pectin can undergo a concentration dependent disaggregation.

Over the last ten years evidence has accumulated that pectin can undergo self disaggregation (1-4). More recently, by size exclusion chromatography (SEC) and determination of number-average degrees of polymerization ($\overline{DP}_n$) from end group titrations (EGT) (5), we have demonstrated self disaggregation over the pH range 3.7 - 7.3 for citrus pectins with degree of methyl esterfication (DM) between 37 and 73%. Moreover, by membrane osmometry (O) and EGT we have shown that protonated and neutralized pectins form metastable aggregates which can be dissociated by heat activation and that these activated pectins undergo concentration dependent disaggregation (6,7). Furthermore, EGT gives the $\overline{DP}_n$ of pectin monomer whereas osmometry gives the $\overline{DP}_n$ of aggregated pectins. All but protonated pectins with low DM (35 or 37%) exhibited disaggregation with a steep concentration dependence. Thus, neutralized pectins and protonated pectin with medium and high DM gave van't Hoff plots which exhibited a minimum at about 0.1 g/dl. Furthermore, the osmotic data could be extrapolated to π/c values obtained from EGT (Figure 1). Similar behavior by proteins has been interpreted as that of a nonideal dissociating system (8). In the case of the pectins, van't Hoff plots appear linear and nonideal above 0.1 g/dl (i.e., they have a positive slope). In the past, the $\lim_{c \to o} (\pi/c)$ for the linear portion of the curve has been used to obtain $\overline{DP}_n$ from membrane osmometry. Here we obtain number-average ($\bar{R}_{gn}$), weight-average ($\bar{R}_{gw}$) and z-average ($\bar{R}_{gz}$) RMS of radii gyration from high performance size exclusion chromatography (HPSEC). By assuming rod-like structure we obtain $\overline{DP}_n$ values from corresponding $\bar{R}_{gn}$ values. These are compared with $\overline{DP}_n$ values from end group analysis and osmometry.

Experimental

Materials. Commercial citrus pectins with degree of methyl esterification (DM) 35, 58-60, and 70 were gifts from Bulmers Limited, Hereford, England. Two other citrus pectin samples DM 37 and 72-73 were manufactured by Bulmers but were gifts from Drs. E. R. Morris and M. J. Gidley at Unilever. The DM 57 citrus pectin was a gift from Sunkist Growers, Corona, Ca. One pectin sample was extracted from fresh grapefruit peels, according to standard procedure (9). It had a degree of esterification of 73 and was labeled 73G. Characterization and preparation of samples were as reported previously (5) with minor modification. Samples to be neutralized with NaOH were dissolved in 0.01 M phosphate buffer (pH 6.1) containing 0.1 M EDTA, titrated to pH 7 with 0.1 M NaOH, dialysed against four changes of water over 48 hr., centrifuged for 1 hr. at 30,000 x g to remove insoluble matter and then lyophilized. Protonated samples were dissolved in deionized water (Continental Water Systems) followed by dialysis, centrifugation, and lyophillization weight cut off of 12,000. Dextran standards were from Pharmacia Chemical Co., Piscataway, N.J. The $\bar{M}_w$ values of the dextran standards were as follows: T-500, 5.32×10^5; T-250, 2.53×10^5; T-110, 1.06×10^5; T-70, 7×10^4; T-40, 4.44×10^4; T-20, 2.23×10^4; T-10, 9.3×10^3. Pullulan standards were from Polymer Laboratories, Inc., Amherst, MA. The $\bar{M}_w$ values of the pullulans were 8.53×10^5, 3.80×10^5, 1.86×10^5, 1.00×10^5, 4.8×10^4, 2.37×10^4, 1.22×10^4 and 5.8×10^3. The pullulans had ratios of $\bar{M}_w/\bar{M}_n$ of 1.14, 1.12, 1.13, 1.10, 1.09, 1.07, 1.06, and 1.07 respectively.

HPSEC

Apparatus, sample preparation and chromatographic conditions were as reported previously (5) with the following modifications. High performance size exclusion chromatography was performed either on a Waters E-1000 µ-Bondagel column (30 x 0.39 cm I.D.) or an E-linear µ-Bondagel column (30 x 0.39 cm I.D.). Twenty µl of a 0.3 mg/ml sample were injected. Mobile phase was either 0.05 or 0.1M NaCl. The solvent in the reservior was stirred with a magnetic stirrer and the column was wrapped with a soft foam insulator. The chromatograph was kept in a constant temperature room at 23±1°C. Flow rates were measured by an air bubble injected into a calibrated measuring pipette connected to the exit line of the chromatograph (10). The pump was set at a nominal flow rate of 0.5 ml/min. Long term flow rates were measured to be within ± 2% of the nominal value. Over any 8 hour period, flow rates were precise to ± 0.3%. Generally, peak maxima for 3 consecutive runs agreed within 2 seconds.

Peaks emerging from the size exclusion column were detected by refractive index and UV absorbance at 206 nm. Analog signals were digitized at a rate of 150 points per minute and in a remote location by a modcomp 7861 minicomputer, equipped with an analog input subsystem.

To obtain averaged root-mean-square radii of gyration, partition coefficients (K_{av}) were transformed point by point to R_g values as appropriate integrals were summed. Integrations were by a trapezoidal algorithm. Transformations were obtained from the following calibration curves:

$$K_1 > K_{av} \quad \ln(Y) = a_0 + a_1 K_{av} \tag{1}$$

$$K_1 < K_{av} < K_2 \quad \ln(Y) = b_0 + b_1 K_{av} + b_2 K_{av}^2 + b_3 K_{av}^3 \tag{2}$$

$$K_{av} > K_2 \quad \ln(Y) = c_0 + c_0 K_{av} \tag{3}$$

Here, Y is R_g. With the aid of equations 1-3, the best regression line is fitted using values of Y and K_{av}. The constants a_0, a_1, c_1 and c_0 are constrained to make the calibration curve and its first derivative continuous at K_1 and K_2. The boundaries K_1, and K_2 are chosen to minimize the sum of the residuals squared. The constants, b_0, b_1, b_2 and b_3 governing that portion of the calibration curve with $K_1 < K_{av} < K_2$ was obtained by non linear regression using the Gauss Newton algorithm. For E-1000 columns, the calibration curve is a cubic polynomial with straight lines at the ends, whereas E-linear calibration curves are cubic polynomials.

For the narrow pullulan molecular weight standards, partition coefficient values corresponding to the peak maximum of the differential refractive index trace (ΔRI) of the chromatogram were correlated with the z-average radii of gyration ($\bar{R}_{gz}$) obtained from the literature (11). For each broad dextran standard, integral distribution curves supplied by the manufacturer gave weight percentage values corresponding to $\bar{M}_w$, the weight-average molecular weight of the standard. Weight percentages were equated with area percentages from the ΔRI trace of the corresponding dextran chromatogram, so that K_{av} on the chromatogram could be correlated with $\bar{M}_w$. By utilizing a combination of $\bar{R}_{gz}$ values from light scattering and viscosity measurements (5), $\bar{R}_{gz}$ values were obtained which correspond to the $\bar{M}_w$ values for each dextran standard. Thus, for each dextran standard, K_{av} values could be correlated with $\bar{R}_{gz}$ values through $\bar{M}_w$.

Membrane Osmometry

Number average molecular weight ($\bar{M}_n$) by osmotic pressure measurements has been described previously (12). The osmometer cell was thermostatted at 35± 0.1°C and the solvent was 0.047 M in NaCl and 0.003 M in NaN_3. All samples were immersed in boiling water for 10 minutes, quenched and stored at 35° C for a minimum of 3 days prior to serial dilution for osmometry.

End Group Titration

The $\bar{M}_n$ of pectins was determined also by the reaction of sodium chlorite with aldehyde end groups. This method was developed for polysaccharides (13) and modified specifically for pectins (14). The chlorite reaction was allowed to proceed for a minimum of 16 hrs. in order to obtain results which were independent of whether end group standards were galacturonic acid or rhamnose.

Results and Discussion

Calibration of Columns. Figure 2 demonstrates that branched dextrans which are relatively compact and linear pullulans which are relatively extended in solution (11) will co-elute on μ-Bondagel columns if they have the same R_g. Furthermore, for the broad dextran samples, the partition coefficient at which $\bar{M}_w$ elutes correlates well with the RMS radius of gyration from light scattering. Thus, we have demonstrated "Universal calibration" (15) for these

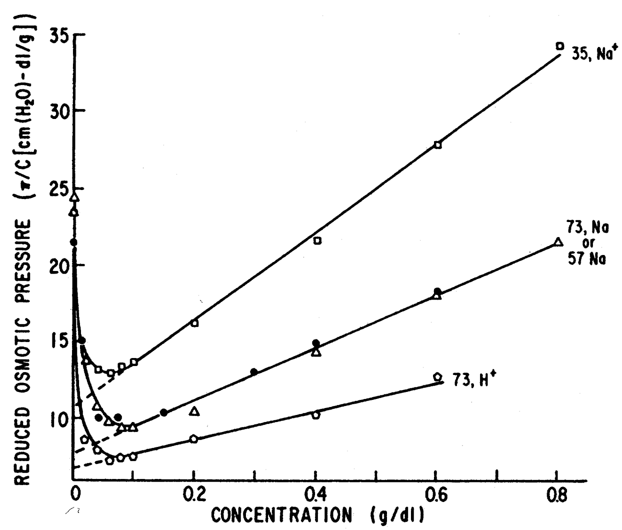

Figure 1. van't Hoff plots demonstrating concentration dependence of pectin dissociation. Values at intercept calculated from end group titrations.

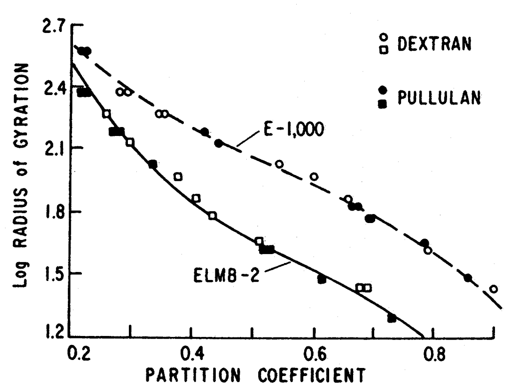

Figure 2. Universial calibration curves for mixture of broad and narrow distributions of polysaccharides in 0.1M NaCl mobile phase.

two classes of polysaccharides without fractionating the commercially available and relatively inexpensive dextran standards (11). On the basis of these results, we have assumed that pectins with R_g values identical to that of dextran or pullulan standards will co-elute.

Table I contains number- ($\bar{R}_{gn}$), weight-, ($\bar{R}_{gw}$), and z-average, ($\bar{R}_{gz}$), radii of gyration. These are defined by equations 4-6

$$\bar{R}_{gn} = \sum_i C_i \;/\; \sum_i (C_i/R_{gi}) \tag{4}$$

$$\bar{R}_{gw} = \sum_i C_i\, R_{gi} \;/\; \sum_i C_i \tag{5}$$

$$\bar{R}_{gz} = \sum_i C_i\, R_{gi}^2 \;/\; \sum_i C_i\, R_{gi} \tag{6}$$

Where R_{gi} is the radius of gyration of species i and C_i is its concentration.

For each DM, $\bar{R}_{gn} < \bar{R}_{gw} < \bar{R}_{gz}$, which is the expected order. Interestingly, for the radius of gyration at peak position (R_{gp}) (maximum concentration of pectin), $\bar{R}_{gn_o} < \bar{R}_{gp} < \bar{R}_{gw}$ for Rgp < 135 Å whereas $\bar{R}_{gw} < \bar{R}_{gp} < \bar{R}_{gz}$ for R_{gp} > 247 Å. Typical chromatograms are shown in Fig. 3.

As indicated by Table I, pooling data without regard to column type, salt concentration in the mobile phase, or pectin form gave data with a standard deviation ranging from 3 - 15% of the mean for the number-, weight- or Z-average R_g. In case of the number average radius of gyration, the standard deviation ranged from 3.5 - 10%. Since, it was our intention to compare molecular weight and size values from SEC with those from end group titrations, and osmometry, (comparisons of log $\bar{R}_{gn}$ reduced the heterogenity in variance (16)) values were analyzed for variance at the ($p \leq 0.05$) confidence level. Such analysis was used to determine if R_g values were affected significantly by: (1) the concentration of salt in the mobile phase; (2) whether the form of the carboxylate ion was hydrogen or sodium; (3) the pore size distribution (psd) of the HPSEC columns. No global trends were identified because of interactions between combinations

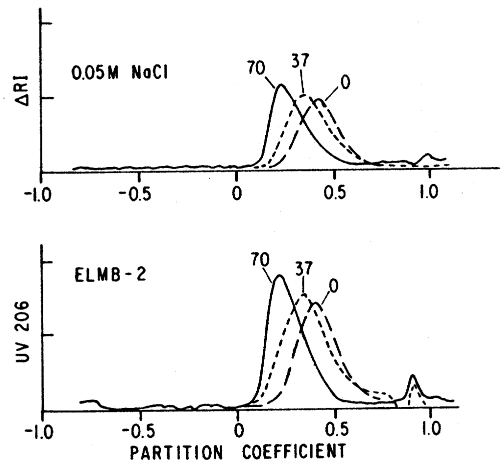

Figure 3. Typical chromotograms for pectins with 0, 37 and 70% methylation esterfication.

TABLE I RADIUS OF GYRATION (R_g, Å) FOR PECTINS[1]

DM \ R_g	NUMBER-AVERAGE	WEIGHT-AVERAGE	Z-AVERAGE	PEAK POS.
0	51.7 ± 2.2	65.2 ± 4.1	84.5 ± 11	53.7 ± 4.2[2]
35	99.6 ± 3.8	144 ± 3.0	212 ± 7.8	123 ± 11
37	70.5 ± 2.2	105 ± 2.9	165 ± 12	81.5 ± 8.2
57	131 ± 9.4	207 ± 17	309 ± 40	258 ± 28
58-60	124 ± 4.3	194 ± 4.5	288 ± 11	252 ± 33
70	126 ± 6.0	201 ± 10	301 ± 12	247 ± 35
72-73[3]	123 ± 12	206 ± 11	314 ± 12	292 ± 35
73G	101 ± 3.3	149 ± 4.0	225 ± 15	135 ± 31

[1] DATA AVERAGED OVER MOBILE PHASE CONCENTRATION (0.05M & 0.1M NaCl), OVER FORM (ACID & NEUTRALIZED) AND OVER COLUMN (E-1000 AND E-LINEAR) 24 DETERMINATIONS.

[2] STANDARD DEVIATION OF POOLED DATA AS IN (1).

[3] DATA FOR E-1000 COLUMN ONLY (12 DETERMINATIONS).

of mobile phase concentration, carboxylate counter ion and column psd. In another approach, at constant degree of methylation, $\bar{R}_{gn}$ values were separated at the ($P \leqslant 0.05$) confidence level through differences in their logs by the Bonferroni LSD method (17). Thus within any horizontal row in Table II, means not followed by the same letter were significantly different at the ($p \leqslant .05$) confidence level. At constant column type and salt concentration only 1/3 of the comparisons in Table II gave significant differences in $\bar{R}_{gn}$ with carboxylate counter ion (e.g. Na against H). Comparisons revealed that differences due to carboxylate counter ion were equally divided between the two column types. Generally at each DM, values of $\bar{R}_{gn}$ for the E-1000 columns tended to cluster around values $\bar{R}_{gn}$ averaged over column type, salt concentration in the mobile phase and counter ion form (i.e. the value of $\bar{R}_{gn}$ in Table I). Multiple letters indicate multiple overlap in means for the E-linear columns, $\bar{R}_{gn}$ values at each DM tended to be higher then $\bar{R}_{gn}$ values for E-1000 columns if salt mobile phase concentration was 0.05 M whereas $\bar{R}_{gn}$ values tended to be lower than E-1000 values of $\bar{R}_{gn}$ if NaCl in the mobile phase was 0.1 M. If one assumes a rod-like model for pectin it is readily shown (12) that the degree of polymerization DP is related to R_g by equation 7.

$$R_g = DP \times h \; / \; \sqrt{12} \tag{7}$$

Here h is the virtual bond length, the length of a monomer unit projected along the x axis, provided the pectin rod is parallel to that axis. If one replaced DP with the ratio of polymer molecular weight M to monomer residue weight, M_o, equation 8 is obtained.

$$R_g = M \times h \; / \; \sqrt{12} \times M_o \tag{8}$$

Substituting equation 8 into equation 4, yields equation 9.

$$\bar{R}_{gn} = (h \; / \; \sqrt{12} \times M_o) \; (\sum_i c_i \; / \; \sum_i \; (c_i \; / \; M_i) \tag{9}$$

TABLE II PECTIN NUMBER AVERAGE RADIUS OF GYRATION (Å)[1]

COLUMN	ELMB[2] - 2				E - 1000				
CONC. NaCl	0.05M		0.1M		0.05M		0.1M		
COUNTER ION / DM	H	Na	H	Na	H	Na	H	Na	SD.[3]
0	55.9b[4]	49.6bc	44.6c	31.9d	54.8b	72.8a	51.8b	52.4b	2.2
35	109a	113a	93.8bc	80.4d	86.6cd	108a	101ab	105a	3.8
37	79.2a	81.9a	59.6c	57.1c	72.1b	69.9b	73.6b	70.9b	2.2
57	152ab	159a	108cd	89.8d	141ab	136ab	136ab	128bc	9.4
58-60	139ab	144a	115d	94.1e	110d	127c	132bc	128c	4.4
70	148ab	145abc	105d	84.8e	107d	133bc	126c	158a	6.0
72-73	- - -	- - -	121a	106b	133a	141a	133a	128a	12.0
73G	110ab	106ab	113a	84.9d	111ab	104b	106ab	95.3c	3.3

1 TRIPLICATE DETERMINATION
2 E-linear μ-Bondagel
3 POOLED STANDARD DEVIATION OF VALUES IN ROW
4 MEANS WITHIN ROW WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT AT 95% CONFIDENCE LEVEL BY BONFERRONI LSD METHOD.

or

$$\bar{R}_{gn} = h \times \bar{M}_n \;/\; \sqrt{12} \times M_o \tag{10}$$

Finally

$$\bar{R}_{gn} = h \times \overline{DP}_n \;/\; \sqrt{12} \tag{11}$$

Similar equations could be derived for weight- and Z-average molecular weights respectively. According to equation 11, molecules with $\bar{M}_n$ have a radii of gyration equal to $\bar{R}_{gn}$. This result cannot be generalized to macromolecules which are not rod-like (18).

A test of equation 11 as the model for pectin is to measure the ratio $\bar{R}_{gn}/\overline{DP}_n$. According to equation 11, this characteristic parameter for rods should be dependent on h. If we take the literature value (19), of 5 Å for pectin, the theoretical characteristic parameters for pectins would be 1.44. In Table III, we have calculated characteristic parameters (CP) from the ratio of $\bar{R}_{gn}$ from SEC and $\overline{DP}_n$ from end group titration. Values for $\bar{R}_{gn}$ were pooled data from the two type columns with 0.05 M NaCl in the mobile phase. Characteristic parameter values for protonated and Na forms of the carboxylate were not pooled. Interestingly in all cases but the grapefruit pectin, CP exceeds the theortical value of 1.44. Values in Table III may exceed 1.44 because $\bar{R}_{gn}$ from SEC is that of aggregated pectins whereas $\overline{DP}_n$ from end group titrations is "monomeric" pectin. At constant DM, in all but one case in Table III, differences in characteristic parameter between the sodium and protonated forms are 10% or less.

From equation 11 and $\bar{R}_{gn}$ from size exclusion chromatography (SEC), it is possible to calculate the $\overline{DP}_n$ of the backbone residues in pectin. The results of these calculation appear in Table IV under the heading SEC-1. Since pectins contain about 22% neutral sugars side chains, dividing the $\overline{DP}_n$ values under SEC-1 by 0.78 yields the $\overline{DP}_n$ values for the entire pectin molecule. The results of those calculations appear under the heading SEC-2 in Table IV. Values of $\bar{R}_{gn}$ were obtained by combining data from the two type of columns and the two pectin forms. Here, 0.05 M NaCl was the mobile phase. For

TABLE III CHARACTERISTIC PARAMETERS FROM NUMBER AVERAGE[1]

DM	H	NA^+
0	1.8	2.0
35	1.9	2.1
37	2.3	2.3
57	2.2	2.2
58-60	1.9	2.1
70	1.9	2.1
72,73	1.8	2.3
73G	1.2	1.2

[1] POOLED DATA FROM ELMB & E-1000 COLUMNS, SOLVENT 0.05M NaCl

TABLE IV. NUMBER-AVERAGE DEGREE OF POLYMERIZATION ($\overline{DP}_n$) OF PECTINS

METHOD / DM	EGT[1]	SEC-1[2]	SEC-2[2]	OSMOMETRY
0	30	40	40	51[3]
35	51	72	92	126[3]
37	33	52	67	94[3]
57	66	102	131	170
58-60	66	90	116	171
70	68	93	119	174
72,73	60	86	111	190
73G	85	71	91	- -

[1] END GROUP TITRATION.

[2] SIZE EXCLUSION CHROMATOGRAPHY.

[3] NEUTRALIZED PECTINS. REMAINING DATA IS A COMBINATION OF Na & H FORMS.

comparison, we have included values of $\overline{DP}_h$ from end group titrations (EGT) and osmometry (O) for these same pectins. As in the cases of the SEC values, except where noted, $\overline{DP}_n$ for Na and protonated forms were combined. The data of Table IV reveal that $\overline{DP}_n$ (EGT) < $\overline{DP}_n$ (SEC) < $\overline{DP}_n$ (O). The order of $\overline{DP}_n$ values could be predicted from Fig 1, since the concentration at which pectin was measured in SEC was intermediate between 0 and 0.1 g/dl. We note that the osmometry data was measured at 35°C whereas the SEC was obtained at 23°C. Thus, if we assume that pectin aggregates primarily through hydrogen bonds, we could expect that pectin $\overline{DP}_n$ values from osmometry at 23°C would be even larger then those measured at 35°C.

The linearity of the osmotic pressure data above 0.1g/dl (see figure 1) may indicate that above this critical concentration, the "monomeric" pectin concentration is practically constant whereas it changes with total polysaccharide concentration below the critical concentration. Ionic detergents behave in this fashion.

Since the product of $\overline{DP}_n$ and h is the number average length, $\bar{l}_n$, equation 11 permits a calculation of $\bar{l}_n$ by dividing $\bar{R}_{gn}$ from SEC by $\sqrt{12}$. In the case of osmometry, as indicated by figure 1, taking the $\lim_{c \to o} (\pi/c)$ of the linear portion of the curve gives the aggregated molecular weight of pectin. Thus, if one multiplies a corrected $\overline{DP}_n$ by (h), then a maximum value of $\bar{l}_n$ (i.e. $\bar{l}_n$ for end to end aggregation) will be obtained. Corrected $\overline{DP}_n$ is obtained by multiplying $\overline{DP}_n$ by 0.78, the mole fraction of galacturonate in the polysaccharide backbone. For $\overline{DP}_n$ from end group titration, multiplying $\overline{DP}_n$ by 0.78 will give the length of monomer residues in the backbone.

Comparison of number average lengths obtained from end group titration, $\bar{l}_n$ (EGT), and from size exclusion chromatography $\bar{l}_n$ (SEC) (Table V) revealed that pectin length decreased, with decreasing concentration presumably due to disaggregation. Furthermore, the maximum length by osmometry is consistent with the concentration-dependent disaggregation.

In summary, we have demonstrated that by utilizing SEC and a variation of the universal calibration principle, one can measure quantitatively, the radius of gyration of pectin in solution. Furthermore, by assuming simple rod-like structure one can obtain molecular dimensions and weights. Statistical analysis of the data revealed that short term precision was about 3% whereas long term precision was about 10%. This discrepancy is probably due to small changes in retention time induced by mechanisms other than size exclusion. Within a precision of ±10%, differences due to counter ion, salt concentration in the mobile phase or pore size distribution of the column packing are marginal. In the long term, somewhat better precision is obtained with the E-1000 columns than with E-linear columns.

Comparison of molecular dimensions and weights for pectins from the various techniques revealed their magnitude to be in the order end group titration < size exclusion chromatography < osmometry. These results are consistant with the hypothesis that pectin can undergo a concentration-dependent disaggregation. Interestingly, an activation step such as briefly heating is necessary to observe disaggregation in the case of osmometry whereas in the case of size exclusion chromatography shear forces within the column appear to perform the same function.

Acknowledgments

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